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Diagnosis of ductal carcinoma *in situ* (DCIS) has risen 500% in recent years.
However, only a fraction of these lesions progress to invasive cancer, making it is important to identify markers that identify high-risk patients. Critically shortened telomeres give rise to genomic instability both *in vivo* and *in vitro* and thereby drive alterations in gene expression. We have shown that reduced telomere DNA content (TC) was associated with decreased survival breast cancers. These findings demonstrate that alterations in TC may occur early in the neoplastic process and are likely to impact clinical outcome. We hypothesized that TC could be a unique and informative prognostic marker in DCIS. The purpose of this project is twofold: 1) determine if TC can be used to predict clinical outcome in a retrospective study of DCIS, 2) determine if loss of telomeric DNA can induce specific changes in gene expression. To date, a study population has been identified and cases selected. Micodissection and telomere-specific FISH protocols have been optimized to determine TC. Additionally, an *in vitro* model has been developed to examine changes in gene expression related to alterations in TC.

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INTRODUCTION: The widespread use of mammography lead to a 500% increase in the diagnosis of ductal carcinoma *in situ* (DCIS) from 1983 to 1992 (1). However, only a fraction of these lesions progress to invasive cancer making it important to identify tumor markers that discriminate high-risk patients, whose disease is likely to progress, from low risk patients, who can be successfully treated with less invasive modalities. It has been shown that critically shortened telomeres give rise to genomic instability both *in vivo* and *in vitro* (2-5) thereby driving alterations in gene expression. We have recently shown that reduced telomere DNA content (TC) was associated with decreased survival in prostate (submitted) and breast cancers. Remarkably, reduced TC was also associated with survival in tumor-adjacent histologically normal breast tissue (in preparation). Collectively these findings demonstrate that alterations in TC may occur early in the neoplastic process and are likely to impact clinical outcome in cancer. Thus we hypothesized that TC will be a unique and highly informative prognostic marker in DCIS. The purpose of the proposed project is twofold. First, determine if TC can be used to predict clinical outcome in a retrospective study of DCIS. Second, determine if loss of telomeric DNA can induce specific changes in gene expression.

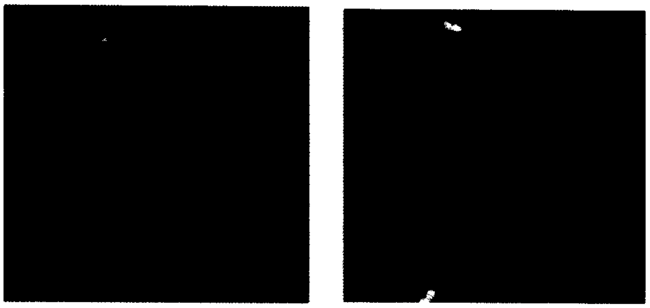
TASKS: The agreed upon tasks to be completed in the statement of work were as follows:

1. Perform a retrospective investigation of the relationship between TC and outcome in DCIS.
2. Perform a prospective investigation of the relationship between TC and gene expression.

PROGRESS RELATIVE TO THE STATEMENT OF WORK: A study population of 60 women diagnosed with ductal carcinoma *in situ* was identified in cooperation with the UCSF Cancer Center Tissue Core. The study population was selected such that half the women developed recurrent DCIS or progressed to invasive breast cancer within 10 years of their original diagnosis. Since tissues housed at the UCSF Cancer Center Tissue Core are collected from multiple sites, it is not always possible to obtain a separate block of genetically matched histologically normal breast tissue. In order to obtain a sample of histologically normal DCIS-adjacent tissue for analysis of telomere content, tissues will be manually separated using an H&E slide as a reference. A trained pathologist will examine the reference slides and regions containing DCIS and histologically normal tissue will be labeled. Whenever possible DCIS lesions will be excised using sterile scalpels, however when tissue heterogeneity excludes this approach, tissue will be microdissected using a laser capture microscope housed in the laboratory. Preliminary analysis demonstrates that these methods, which produce populations enriched for either tumor or normal cells, can yield sufficient DNA for analysis of telomere content.

Recently a new fluorescent *in situ* hybridization method for measuring telomere content (Telo-FISH) has been developed (4, 6). While very similar to the dot-blot method originally proposed for this investigation, Telo-FISH requires one tenth of the starting material and preserves tissue architecture. We have successfully utilized Telo-FISH for other investigations (shown in figure1). Telomere content from patients without recurrent disease will be determined using both methods. If these approaches are comparable, as seems likely, Telo-FISH will be utilized for analysis of tissues from patients with recurrent disease thereby preserving this valuable resource and eliminating the necessity of mechanical separation of the DCIS and DCIS-adjacent histologically normal tissues. It is anticipated that DNA will be extracted and analysis will be performed on the non-recurrent DCIS tissues by December of 2004. Analysis of the recurrent DCIS lesions will be completed following statistical analysis of the variance and distribution of telomere contents in the non-recurrent DCIS tissues.

Figure 1: Representative Images of Telomere-Specific Fluorescent *In Situ* Hybridization

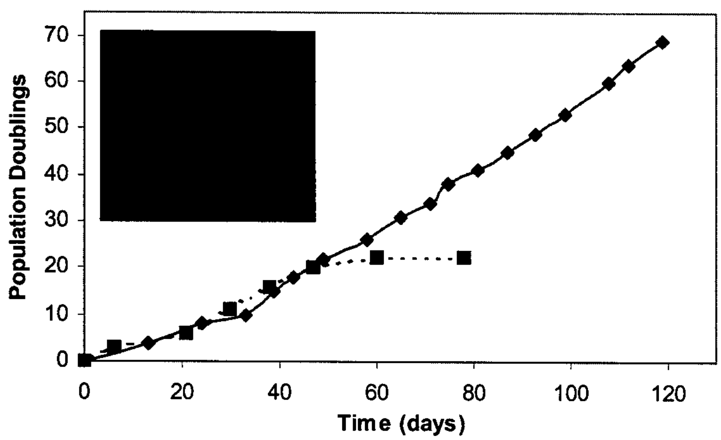


Human breast tissue was hybridized to a Cy3-labeled telomere-specific probe (red). Nuclei were counterstained with DAPI (blue).

It was originally proposed that changes in gene expression related to telomere length be studied *in vivo* by performing microarray analysis on frozen samples of DCIS with differing telomere lengths. However, it has been difficult to obtain sufficient numbers and amounts of tissues. Additionally, the potentially confounding effects changes in gene expression related to the hormonal status of the patient would have made statistical analysis difficult. In order to overcome these limitations an *in vitro* system has been developed. Human mammary epithelial cells (HMEC) were isolated from reduction mammoplasty specimens obtained from women without signs of breast disease. As previously reported, HMEC grow for approximately 20 to 30 population doublings in culture before growth arrest. A subpopulation of HMEC, termed variants or vHMEC, is able to silence p16 through promoter hypermethylation and continue to proliferate and additional 20 to 50 population doublings until reaching a second growth arrest termed agonescence (7). Cells at or near agonescence exhibit the types of genomic changes characterized by malfunctioning telomeres and have shorted telomeres. Since some changes in gene expression observed vHMEC may not be caused by telomere malfunction it is not practicable to use isogenic HMEC as a reference in microarray experiments. In contrast, comparison of expression profiles of vHMEC with short telomeres to vHMEC whose telomeres have been stabilized would be a consequence of changes in telomeric DNA.

To date vHMEC from two different donors have been infected with a green fluorescent protein-human telomerase construct. Introduction of telomerase is believed to immortalize cells through extension of the telomeres. Indeed, vHMEC infected with the GFP-telomerase construct show nuclear localization of telomerase and grow well past parental controls (Figure 2). Cytogenetic analysis is being performed on these cells in order to ensure that both the parental and telomerase cell lines have comparable karyotypes. Telomere content will be analyzed and RNA prepared for microarray analysis in the coming weeks. Additionally, vHMEC isolated from two additional donors will be infected with the GFP-telomerase construct. Genes whose expression is consistently down regulated at least three fold in the vHMEC-telomerase cell lines will be validated using RT-PCR and immunocytochemistry and subsequently examined *in vivo* using immunohistochemistry.

Figure 2: Growth Characteristics of vHMEC Expressing Human Telomerase



vHMEC expressing telomerase continue to proliferate (diamonds) when control cells (squares) reach agonescence. Population doublings are shown (Y axis) vs. days in culture following infection with the GFP-telomerase construct (X axis). Inset: vHMEC showing GFP-telomerase (green) localization in the nucleus (blue).

KEY RESEARCH ACCOMPLISHMENTS:

- Cases of recurrent and non-recurrent DCIS for telomere content analysis have been identified in cooperation with the UCSF Cancer Center Tissue Core.
- DNA yields from manually excised and microdissected breast tissues have been determined.
- The telomere-specific fluorescent *in situ* hybridization (telo-FISH) protocols have been optimized for use in archival paraffin-embedded breast tissues.

REPORTABLE OUTCOMES:

- Primary human mammary epithelial cell lines, which contain the telomerase gene, have been produced.
- Dr. Colleen Fordyce has been supported by this training grant. She has gained expertise in confocal and laser-capture microscopy and culture and infection of primary human cells, and breast pathology and histology.

CONCLUSIONS: At present there is no reliable method to determine which DCIS lesions will remain indolent and which will progress to invasive breast cancer (1, 8, 9). Effective identification of patients with aggressive DCIS lesions would make it possible to match treatment modalities to each patient's disease, thus eliminating unnecessary treatment-related side effects and minimizing treatment-related expense. Telomere content (TC) has potential as a novel prognostic marker in breast cancer and likewise, may predict outcome in DCIS. A retrospective study has been undertaken to examine the relationship between TC and outcome in DCIS. A retrospective patient population composed of women diagnosed with DCIS that did and did not recur has been identified. Tissues from women with non-recurrent disease have been selected. A protocol for microdissection of breast tissues has been optimized. Microdissection produces populations enriched for either histologically normal epithelia or DCIS that will subsequently be analyzed for telomere content using a dot blot assay. A second protocol for measurement of TC, telo-FISH has been optimized. Telo-FISH, requires one tenth the tissue of the dot blot method and preserves tissue architecture. Telo-FISH will be evaluated in comparison to dot blot in the non-recurrent DCIS. If TC determined by telo-FISH is comparable to that determined by dot blot, Telo-FISH would be utilized for analysis of the recurrent DCIS.

Microarray experiments will demonstrate if a change in TC does, as seems likely, induce specific changes in gene expression. Moreover, these experiments may further elucidate which genes are critical and, dependent on the findings in specific aim one, may help identify additional genes involved in cancer development and progression, and therefore potentially identify targets for therapeutics and new tumor markers. Initially it was proposed that TC would be measured in DCIS lesions with different TC. RNA would be purified and utilized for microarray experiments to examine differences in gene expression profiles. However, it has not been possible to obtain sufficient quantities of tissue to satisfactorily complete this objective. Thus and *in vitro* model system was developed. Human mammary epithelial cells were isolated from women without known breast disease and grown in culture. These cells (vHMEC) were infected with a gene for human telomerase, the enzyme that lengthens telomeres. Cells expressing telomerase grow significantly longer than vector controls. To date cells from two donors have been infected with telomerase. vHMEC from two additional donors will be engineered to express human telomerase. RNA will be purified from the cells expressing telomerase and utilized for microarray analysis. Genes identified by microarray analysis will be evaluated *in vitro* using RT-PCR, immunocytochemsitry and western blotting and *in vivo* using immunohistochemistry.

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